

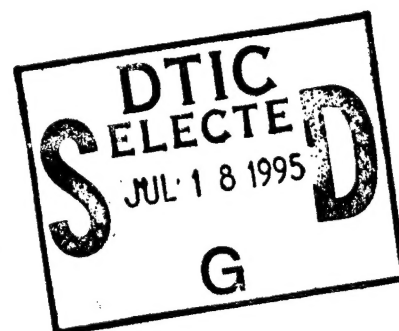
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IN BREAST CANCER

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Kevin J. Cullen, M.D.
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Annual Report
January 1995

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Insulin-like growth factor II as a prognostic variable in breast cancer.

Kevin J. Cullen, PI

Annual Report - January 1995

Introduction

The objective of this project is to examine the role of Insulin-like growth factor II (IGF-II) in interactions between stromal and epithelial cells in human breast cancer. The fundamental hypothesis for this work is that IGF-II produced by breast tumor stroma is an essential and active participant in the process of malignant tumor progression. While the coordinated growth of stromal and epithelial elements is necessary for tumor survival, the specifics of the growth enhancing interactions between these cell types is not clearly defined. We hope that these studies will provide a better understanding of the growth promoting relationships between breast tumor stromal and epithelial cells and may help to identify new therapeutic targets for more rational and effective breast cancer treatment.

In the last decade, a large body of experimental evidence has emerged regarding the importance of peptide growth factors in the regulation of breast cancer and other malignancies.^{1,2} Among the various families of peptide growth factors, the insulin-like growth factors, IGF-I and IGF-II, are part of a group of ligands, receptors and binding proteins which have a significant role in normal development and growth. IGF-I mediates the effects of growth hormone, while IGF-II appears to be an important fetal growth factor with unknown function in adult life.^{3,4,5}

Pre-clinical data

Several groups, including our own, have previously shown that IGF-I and IGF-II are potent mitogens for breast cancer epithelial cells.^{6,7,8,9} We have shown that while surgical breast tumor specimens express both IGF-I and IGF-II on an mRNA level.^{8,10} However, studies of cultured breast tumor epithelial cells as well as *in situ* hybridization studies suggested that the IGF expression was primarily originating in the stroma of the breast rather than in tumor epithelium. IGF-II message appeared to originate in tumor stroma, while IGF-I message arose from stroma in normal breast.^{8,10,11}

Since these data suggested that tumor IGF production originates in stroma rather than epithelium, we examined primary cultures of breast fibroblasts derived from benign and malignant lesions for expression of various growth factors, including IGF-I and IGF-II.¹² The results are summarized on Table 1. The most significant finding from this study is that the majority of fibroblasts derived from malignant lesions expressed IGF-II, while the majority of fibroblasts from benign lesions expressed IGF-I. No tumor

specific differences in gene expression were observed with any of the other growth factors examined. This further supports the notion of a paracrine growth promoting role

Fibroblast	IGF I	IGF II	PDGF A	PDGF B	TGF α	TGF β 1	basic FGF	FGF 5	Source
191	+	-	+	-	-	+	+	+	Red. Mammo.
365	+	-	+	-	-	+	+	+	Red. Mammo.
429	+	-	ND	ND	ND	ND	ND	ND	Red. Mammo.
446	ND	-	ND	ND	ND	ND	ND	ND	Red. Mammo.
879	+	-	ND	ND	ND	ND	+	+	Fibroadenoma
987	+	-	ND	ND	ND	ND	+	ND	Fibroadenoma
999	-	-	+	-	ND	ND	ND	ND	Fibroadenoma
1034	+	-	+	-	-	+	+	+	Fibroadenoma
1097	+	+	ND	ND	ND	ND	+	ND	Red. Mammo.
197	ND	ND	ND	ND	ND	ND	+	+	Tumor
406	+	-	ND	ND	ND	ND	+	+	Tumor
559	-	+	+	-	-	+	+	+	Tumor
788	-	+	+	-	-	+	ND	ND	Tumor
906	-	+	+	-	-	+	+	ND	Tumor
926	-	-	ND	ND	-	+	+	+	Tumor
971	-	+	ND	ND	ND	ND	ND	ND	Tumor
974	-	-	+	-	-	+	+	+	Tumor
977	-	+	+	-	-	+	ND	ND	Tumor
995	-	-	+	-	-	+	+	+	Tumor

Table 1. Growth factor mRNA expression by breast fibroblast cell lines.

Key: IGF-I = Insulin-like growth factor I; IGF-II = Insulin-like growth factor II; PDGF A = Platelet derived growth factor A chain; PDGF B = Platelet derived growth factor B chain; TGF α - Transforming growth factor alpha; TGF- β 1 = Transforming growth factor beta 1; basic FGF = basic Fibroblast Growth Factor; FGF-5 = Fibroblast growth factor 5; ND = not done.

for the insulin-like growth factors in breast lesions, and suggests that IGF-II may be the more important growth promoter in malignant lesions.

Since our results suggested that IGF-II might be serving as a paracrine growth stimulant in breast cancer, we asked whether overexpression of IGF-II by a breast tumor epithelial cell line could confer phenotypic changes associated with malignant progression in breast cancer, such as loss of estrogen dependence for growth. We infected a well differentiated, estrogen-dependent breast tumor epithelial cell line (MCF-7) with a retroviral vector containing the coding sequence for the IGF-II pre-prohormone along with a constitutive CMV promoter sequence. MCF-7 cells do not normally express IGF-I or IGF-II.⁷

All IGF-II overexpressing clones showed marked phenotypic changes associated

with malignant progression i.e. growth on plastic in the complete absence of estrogen, cloning in soft agar without estrogen. Wild-type MCF-7 cells and control cells infected with an irrelevant DNA sequence showed none of these properties.¹³

Finally, we have developed co-culture models which demonstrate that soluble factors produced by tumor derived fibroblasts are markedly mitogenic for breast tumor epithelial cells, and that these mitogenic effects can be inhibited by an antibody which blocks the IGF-I receptor,¹⁴ implying stromal IGF-II is the source of the mitogenic signal.

The present study

Recent work in other centers has suggested that biochemical markers such as c-erb-b2 and cathepsin D can be significant independent prognostic indicators in breast cancer^{15,16} with great potential clinical utility. In light of the laboratory data discussed above, it is therefore reasonable to ask if the same applies to IGF-II.

BODY

Materials and Methods

IGF-II *in situ* hybridization

The *in situ* hybridization protocol was modified from the procedure of Bondy, *et al.*¹⁷

IGF-II Riboprobe : The IGF-II cDNA was kindly provided by Dr. Graeme Bell (Howard Hughes Institute, Chicago, IL). An 833 base pair *Pst*I fragment was sub cloned into a pGEM4 vector (Promega, Madison, WI). The vector was linearized with *RSa*I, providing a template for an antisense riboprobe protecting a 336 base pair mRNA fragment. Labeled antisense RNA was transcribed using T7 polymerase according to the manufacturer (Promega). Labeled sense RNA transcribed using SP6 polymerase was utilized for the negative control. Probes were double labelled with ³⁵S-UTP and ³⁵S-CTP (Amersham- Arlington Heights, IL) to increase specific activity.

In-Situ Hybridization : 5 micron sections were cut from paraffin blocks and mounted on sialinized slides. The sections were deparaffinized and rehydrated through a progressive ethanol series. The sections were digested with 1 mcg/ml proteinase K in 100 mM TRIS-50 mM EDTA for 30 minutes at 37 C. After washing in DEPC ddH₂O, the sections were acetylated for 10 minutes at room temperature (RT) in fresh acetic anhydride, diluted 1:400 in 0.1 triethanolamine, pH:8.0. The sections were then dehydrated in an ascending ethanol series and air dried for 1 hour. The tissues were

hybridized with 3×10^7 cpm/ml labeled antisense RNA in 50% formamide, 10% dextran sulfate, 50mM Tris pH:8, 2.5mM EDTA, 2.5% Denhardt's, 0.2 M NaCl, and 250mcg/ml yeast tRNA. The slides were coverslipped and placed in humid chamber sealed with paraffin at 55°C overnight. After hybridization, the slides were placed in a shaking bath in 4X SSC at room temperature until the coverslips floated off. The slides were then washed in four changes of 4X SSC, five minutes each. Next, the tissues were placed in 50% formamide for 10 minutes at 60°C. The slides were then dipped in 2XSSC, with 2ml 1M DTT, followed by incubation with RNase A 20 mcg/ml in 0.5M NaCl, Tris 10 mM pH8, EDTA 1mM, 2ml DTT. The final washes consisted of decreasing concentrations of SSC (2X, 1X, 0.5X for 5 minutes each at RT, 0.1X for 15 minutes at 50°C and 0.1X to cool). Sections were dehydrated through an ascending ethanol series and the air dried for 1 hour. The sections were then exposed to Hyper film-beta max (Amersham) for 3 days to check the completeness of washing. If further washing was necessary the sections were rehydrated and rewashed. The sections were then exposed to NTB-2 emulsion (Eastman Kodak, Rochester, NY) for three weeks. The emulsion was developed with D19 solution for 4 minutes at 15°C. Developing was stopped with in a solution of 200 ml ddH₂O with 1.33 ml glacial acetic acid, and the slides were fixed in 30% Sodium Thiosulfate for 3 minutes. The slides were washed and placed in ddH₂O for 10 minutes. The sections were then stained with hematoxylin and eosin, dehydrated, and coverslipped.

A time course study of the hybridization reaction showed maximum signal after overnight incubation at 55°C. Background signal increased significantly after this period without increase in specific hybridization signal. Riboprobe synthesized in the sense direction was used as a negative control in all assays. All control samples were noted to have some IGF-II mRNA detected in the smooth muscle walls of blood vessels. This served as an intrasample control. Any breast tumor specimens which did not show some specific signal in blood vessel walls was excluded from subsequent analysis. For each sample, 10 high power fields within the tumor were analyzed, and specific clusters of silver grains were counted, corresponding to a cell which expressed IGF-II mRNA. Based on these counts, the tumors were divided into tumors were divided in four groups based on the level of IGF-II mRNA signal: 0 (absent), + (low), ++ (intermediate) and +++ (high). For some of the subsequent statistical analysis, the negative and + scores were grouped together, as were the ++ and +++ scores.

IGF II Immunohistochemistry

Anti IGF-II antibody: We used a polyclonal rabbit antibody raised against intact human recombinant IGF-II peptide, generously provided by Dr. Judith Heisserman (Lilly Research Labs, Eli Lilly Corporation, Indianapolis, IN). Antibody specificity was confirmed by ELISA, which showed less than 10% cross reactivity with recombinant IGF-I and no reactivity with recombinant epidermal growth factor (EGF). Additionally, the antibody was affinity purified using intact recombinant peptide. Affinity purified

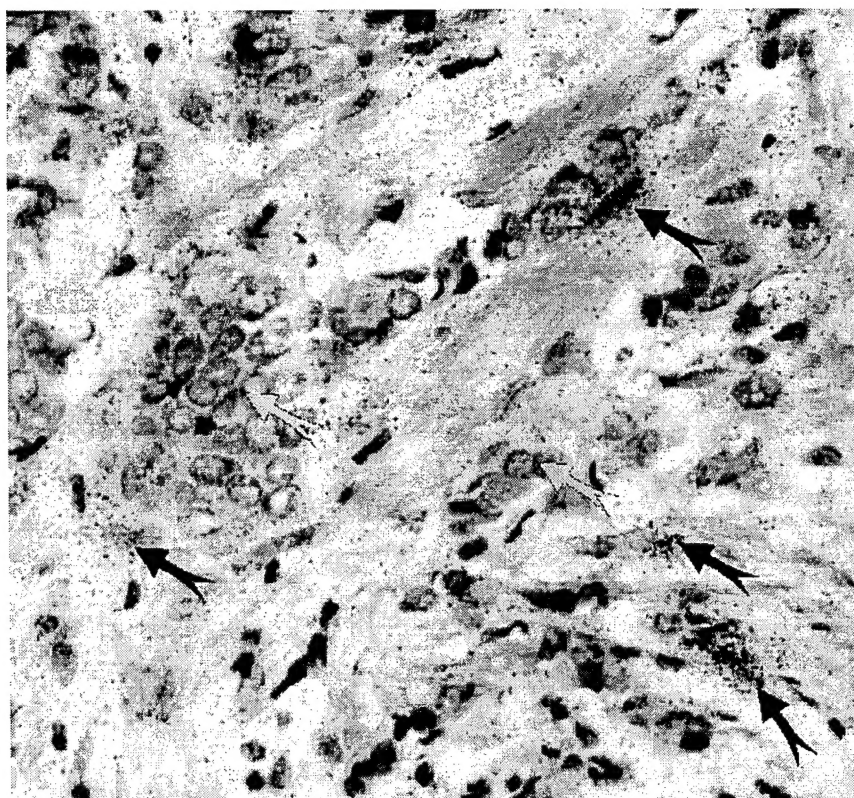
antibody showed identical staining and reactivity in the ELISA as that obtained with polyclonal antibody.

Immunohistochemical staining: Paraffin sections were deparaffinized and rehydrated through progressive ethanol series. The slides were washed in PBS and incubated with normal goat serum for 20 minutes at room temperature. The primary IGF-II antibody was diluted 1:2000 in PBS containing 1% BSA and 1% sodium azide and incubated on the tissue sections at 4°C overnight. The slides were then rinsed twice in PBS for 3 min. each wash. The reaction was visualized with Biogenix multilink system (Biogenix - San Ramon, CA). The streptavidin/alkaline phosphatase linked secondary antibody was incubated for 20 minutes at room temperature. The labelled secondary antibody was detected with the chromagen fast red.

Results

Representative photographic results of the in situ hybridization and immunohistochemistry are presented below.

Figure 1 - IGF-II *in situ* hybridization in an infiltrating ductal breast carcinoma. Silver grains representing IGF-II mRNA expression are seen over specific stromal cells (black arrows) but not over tumor epithelial cells (white arrows). This indicates that IGF-II expression in most breast tumors is stromal in origin and exerts a paracrine effect on tumor epithelium.



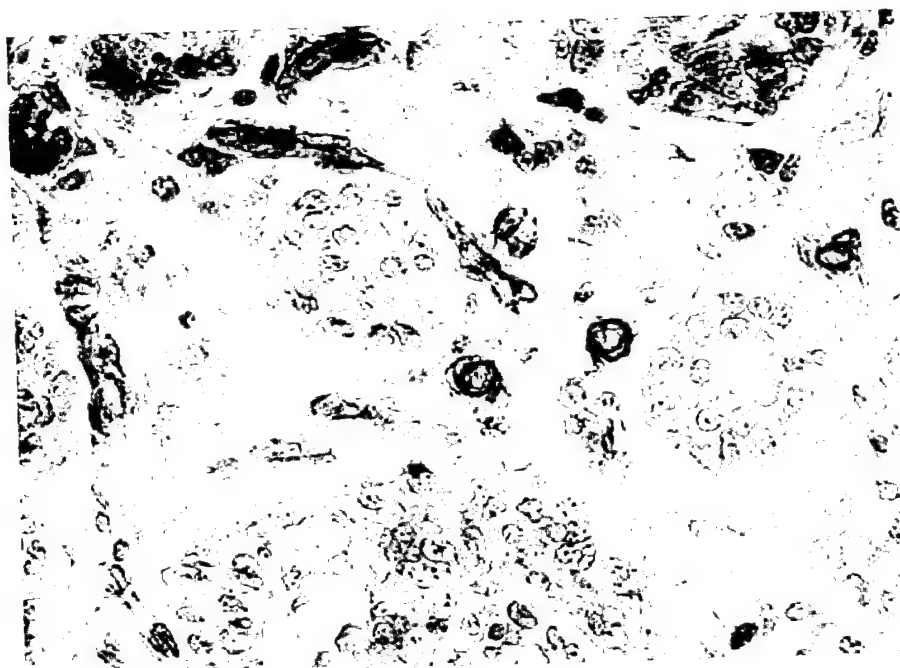


Figure 2. IGF-II immunohistochemistry in an infiltrating ductal carcinoma. IGF-II expressing stromal cells stain red, while tumor adjacent tumor epithelium shows little discernable staining.

To date, we have processed over 300 surgical specimens for both IGF-II *in situ* hybridization and immunohistochemistry. These include over 70 cases from a separate institution, which will be used to validate the data from the Georgetown cases. All cases have been pathologically scored. Complete correlation with clinical data has been finished for the first 80 of these. We are currently scoring the final cases, and completing the compilation of clinical information for the most recent additional cases, and are correlating the clinical staging and outcome data with the IGF-II scoring.

IGF-II *in situ* and immunohistochemistry scores for the cases analyzed so far are as follows:

IGF-II Score	<i>In situ</i> hybridization	Immunohistochemistry
Negative	53 (23%)	39 (16%)
Low (+)	77 (33%)	56 (23%)
Moderate (++)	68 (29%)	103 (43%)
High (+++)	36 (15%)	42 (18%)
Total	234	240

Table 2. IGF-II *in situ* hybridization and immunohistochemistry scores for 240 consecutive breast cancer specimens. The distribution of both scores is similar, although the average score for immunohistochemistry is somewhat higher than for *in situ* hybridization, possibly reflecting a greater sensitivity for that technique.

While we have now performed immunohistochemical and *in situ* analysis on 300 specimens, we have completed clinical correlation and statistical analysis on the first 80 specimens.

Nuclear Grade and lymph node involvement

The distribution of nuclear grade was as following: 18(22.8%) grade 1, 39 (49.4%) grade 2, 22 (27.8%) grade 3. Axillary lymph node involvement was found in 47 cases (59.5%)

ER and PR status

Thirty two (40.5%) out of 79 tumors were ER, PR positive (ER+PR+), 17 (21.5%) ER+PR-, 7 (8.9%) ER-PR+ and 23 (29.1%) ER-PR-. The nuclear immunostaining was located only in malignant epithelial cells (Fig.1). No ER or PR staining presence was seen in the stroma of the tumor.

Proliferating activity

High proliferating activity (> 10% immunoreactive tumor cells) was found in 24 of 77 cases (31.1%).

ras, c-erb-B2, p53

Thirty six (46.7%) of 77 tumors were positive for p21 protein, 20 (25.8%) for p185 and 12 (15.6%) for p53.

IGF-II in situ

Two tumors had no significant (0)IGF-II and 35 had low(+) IGF-II mRNA expression, 20 and 18 showed moderate(++) or high(+++) IGF-II mRNA content, respectively. Thus, the 0, + group included 37(49.4%) cases and the ++, +++ group comprised 38 cases (50.6%). The IGF-II mRNA signal was generally located in the stromal component of the tumor, usually tightly adjacent to the malignant epithelial cells. (Fig.2) Furthermore,

higher IGF-II mRNA expression was present in the loose stroma rather than in dense (established) stroma.

Correlation study

IGF-II in situ vs menopausal status : no statistical relation was found between IGF-II mRNA and menopausal status.

IGF-II in situ vs node involvement and tumor size: no relationship was found between IGF-II mRNA content and node status or number of metastatic nodes. Similarly no relationship was present with tumor size.

IGF-II in situ vs nuclear grade : no relationship was found between IGF-II expression and nuclear grades of the tumors.

IGF-II in situ vs ER and PR . Higher IGF-II mRNA was detected in ER+PR+ tumors (67.8%) than in the others ER/PR classes ($p < 0.05$) (Tab.1). When ER and PR were separately examined, no relationship was found between ER and IGF-II expression. Opposite results were obtained for PR: twenty-four out of 35 (68.6%) PR+ tumors had high IGF-II expression and 11(31.4%) low or absent IGF-II mRNA content. In contrast, most tumors which expressed undetectable or low IGF-II levels (65%) were also PR-. The relationship between PR status and IGF-II expression was significant ($p < 0.01$) (Tab.2). Furthermore, IGF-II mRNA content was directly related with both percentage values ($p = 0.006$) and staining intensity ($p = 0.003$) of PR positive cells.

ER and PR vs stromal proliferation : Twenty-two of 36 (61.1%) tumors with marked stromal proliferation were ER+PR+. In contrast, ER+PR+ was detected in 10 out of 43 (23.2%) tumors with faint stromal proliferation. The relationship between ER, PR status and desmoplasia were significant ($p < 0.01$). The separate analysis of ER and PR showed that this correlation was present only for PR ($p < 0.01$). In fact, 28 out of 36 (77.8%) specimens with marked stromal proliferation were PR+ and 8 (22.2%) were PR-. Interestingly, the relation between PR and stromal proliferation was independent of IGF-II mRNA expression as shown by multi variate analysis. Finally, no relationship was found between desmoplasia and ER status.

IGF-II vs proliferating activity:: IGF-II mRNA expression was not related with proliferating activity assessed by Ki 67

IGF-II vs oncogene protein products: p53, p21 and p185 were not related to IGF-II mRNA expression

IGF-II and clinical outcome : Generally, IGF-II mRNA was not related to survival.

However the expression of IGF-II mRNA modified the predictive value of both ER and PR. In fact, the four yr survival of the- patients with high tumor IGF-II mRNA content was significant lower in the absence of ER ($p=0.007$) or PR ($p=0.01$) Finally, the presence and the content of IGF-II mRNA did not modify the prognosis in the patients with positive nodes.

CONCLUSION

Our data indicate that IGF-II mRNA and protein are expressed in the stroma of the majority of breast cancers. Correlative analysis of the first 80 cases indicates that IGF-II mRNA may confer a poor prognosis in estrogen receptor negative patients.

At the present time we have processed over 300 clinical specimens. We are in the process of finishing scoring for those cases, and correlating the results of our analysis with the clinical data on each case. The results discussed above on the first 80 patients for whom we have completed this analysis show that IGF-II mRNA and protein may alter the prognosis in a subset of patients.

We will complete the comparative analyses on the patients we have processed prior to examining any further cases. If the statistical analysis indicates that we need more cases to answer our initial hypotheses, we will add cases as necessary. At the end of the original time period for this contract, we requested an extension, as we had not completed our analysis. Presently, we have completed immunohistochemical and in situ hybridization analysis of 300 cases.

We are behind schedule in logging the corresponding clinical data for these cases, primarily because of the resignation of the data management person at the Lombardi Cancer Center who was responsible for the maintenance of the data base used for this analysis. However, at the present time we are completing this data analysis in our own laboratory. All "bench work" has been completed, and we are actively entering the corresponding clinical data for the correlative studies and statistical analysis. The data from these cases will provide sufficient power to test our fundamental hypothesis accurately. We regret that the final data analysis is behind our original schedule outlined when this proposal was submitted.

APPENDIX

Table 3: Relationship between ER/PR status and IGF-II *in situ*

IGF-II <i>in situ</i>		0	+	++	+++
ER/PR status	+/+	1	8	10	9
		9			19
	others	1	27	10	9
		28			19

X²: 4.25, $p < 0.05$

Note: "Others" include ER+PR-, ER-PR+, ER-PR- breast tumors

Table 4: Relationship between PR and IGF-II *in situ*

IGF-II <i>in situ</i>		0	+	++	+++
PR status	-	1	25	8	6
		26			14
	+	1	10	12	12
		11			24

Note: The relationship between PR positivity of epithelial cancer cells and the intensity of the stromal IGF-II mRNA expression was statistically significant: X: 7.14, $p < 0.01$.

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